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The minute virus of mice exploits different endocytic pathways for cellular uptake

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ABSTRACT

The minute virus of mice, prototype strain (MVMp), is a non-enveloped, single-stranded DNA virus of the family *Parvoviridae*. Unlike other parvoviruses, the mechanism of cellular uptake of MVMp has not been studied in detail. We analyzed MVMp endocytosis in mouse LA9 fibroblasts and a tumor cell line derived from epithelial–mesenchymal transition through polyomavirus middle T antigen transformation in transgenic mice. By a combination of immunofluorescence and electron microscopy, we found that MVMp endocytosis occurs at the leading edge of migrating cells in proximity to focal adhesion sites. By using drug inhibitors of various endocytic pathways together with immunofluorescence microscopy and flow cytometry analysis, we discovered that MVMp can use a number of endocytic pathways, depending on the host cell type. At least three different mechanisms were identified: clathrin-, caveolin-, and clathrin-independent carrier-mediated endocytosis, with the latter occurring in transformed cells but not in LA9 fibroblasts.

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Introduction

The cellular entry mechanisms of many viruses are well known, as these mechanisms usually determine viral infectivity and offer potential anti-viral targets. However, the early infection events of the parvovirus minute virus of mice, prototype strain (MVMp), are only recently starting to be elucidated. MVMp is a small (about 26 nm in diameter), non-enveloped virus with a very small (5 kb) single-stranded DNA genome that carries only two open reading frames (ORFs). The 5′ ORF encodes the structural proteins VP1 and VP2, while the 3′ ORF encodes two non-structural proteins NS1 and NS2 (reviewed by Cotmore and Tattersall, 2006). VP1 and VP2 are produced by alternative splicing of the viral mRNA, and therefore VP1 contains the complete sequence of VP2, as well as a 143-residue unique N-terminal sequence. VP1 and VP2 compose the icosahedral capsid that protects the viral genome from environmental extremes and mediates viral interaction with cell surface receptor(s). NS1, the first viral protein to be expressed during infection, is a multifunctional nuclear phosphoprotein, with roles ranging from initiating viral DNA transcription to cell cycle arrest, DNA-damage response, and release of progeny virions (reviewed by Nuesh and Rommelaere, 2014). NS2 is essential for viral DNA amplification (Ruiz et al., 2011) and for the egress of progeny virions from the nucleus of infected cells (Eichwald et al., 2002).

MVMp attaches to its target cells via sialic acid on an unknown glycoprotein receptor and subsequently enters the endosomal pathway. After endocytosis, MVMp escapes from endocytic compartments into the cytosol by means of the enzymatic action of a phospholipase A2 (PLA2) motif in the unique region of VP1 (Farr et al., 2005). Although the mechanism of endosome escape has been elucidated, the cellular internalization route of MVMp and the cellular factors involved in this process await further studies. Yet, the endocytic mechanisms used by other parvoviruses have been well characterized. For example, canine parvovirus and feline panleukopenia virus use the transferrin receptor to enter their host cells by clathrin-mediated endocytosis (CME) (Parker and Parrish, 2000; Parker et al., 2001); parvovirus B19 also enters via CME (Quattrocchi et al., 2012). Recent studies have shown that, in addition to CME, parvoviruses can use several other endocytic pathways. For example, adeno-associated virus type 2 (AAV-2) uses clathrin-independent carriers (CLICs) (Nonnenmacher and Weber, 2011), AAV-5 uses caveolae-dependent endocytosis (Bantel-Schaal et al., 2009), and porcine parvovirus uses both CME and macropinocytosis (Boisvert et al., 2010). Thus, although parvoviruses share some general features, the literature on their routes of cellular uptake does not allow for mechanistic generalizations.

The cellular internalization mechanism used by MVMp has not been studied in detail. In principle, MVMp could use CME, as canine parvovirus does; caveolae-dependent endocytosis, as AAV-5 does; CLICs, as AAV-2 does; or any of the various novel endocytic

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mechanisms that have been identified recently and are known to be used by several other viruses (Mercer et al., 2010; Schelhaas, 2010). The identification of the endocytic pathway used by MVMp is important to reconstitute its mechanism of infection completely and may help us to understand the oncotropic characteristics of this virus.

We used electron microscopy (EM), immunofluorescence (IF) microscopy, and fluorescence-activated cell sorting (FACS) in combination with inhibitors of various endocytic pathways to elucidate the endocytic mechanisms used by MVMp. We found that MVMp is endocytosed from the base of filopodia and from cell-extracellular matrix (ECM) contact sites at the leading edge of migrating cells via at least three different endocytic pathways: clathrin-, caveolin-, and CLIC-mediated endocytosis.

Results

Electron microscopy of MVMp cellular uptake

To elucidate the MVMp uptake pathway, we performed EM analysis of two different cell lines infected with MVMp: LA9 mouse fibroblasts, a model for the study of MVMp infection, and mouse mammary cells transformed with polyomavirus middle T antigen (PyMT cells, Granovsky et al., 2000), highly migrating cells that are more susceptible to MVMp infection than non-motile cells (Garcin and Panté, 2014). To visualize a large number of virions in endocytic compartments close to the plasma membrane, we performed a short-term MVMp endocytosis assay by incubating the cells with MVMp at 4 °C for 15 min, and then switching the cells to 37 °C for 5 min (PyMT) or 10 min (LA9). After sample preparation for EM, ultrathin *en-face* cell sections (i.e., sections parallel to the cell monolayer) were positively stained and observed by transmission EM. As illustrated in Fig. 1, both LA9 and PyMT cells displayed MVMp-containing endocytic vesicles at the base of filopodia and near the plasma membrane. Control cells mock incubated with medium instead of MVMp lacked any virion-containing vesicles (Fig. S1). Immunogold labeling using an anti-MVMp capsid antibody confirmed that the round, electron-dense particles of about 26 nm in diameter observed in our electron micrographs were indeed MVMp particles (Fig. S2). The results shown in Fig. 1 are consistent with our recently published finding that MVMp particles cluster at the leading edge of migrating cells rapidly after binding to the cell surface (Garcin and Panté, 2014). These results indicate that MVMp could be endocytosed at the leading edge of migrating cells.

Close inspection of the MVMp-containing vesicles by EM in both cell types revealed multiple MVMp particles internalized in coated vesicles, which exhibited all the hallmarks of clathrin-coated vesicles (Fig. 2A, top panels). We also detected single MVMp particles in small vesicles and in flask-shaped invaginations typical of caveolae (Fig. 2A, bottom panels), and in elongated tubular compartments associated with CLICs (Fig. 2B). The latter structure was found only in PyMT cells. These results clearly indicate that MVMp uses several endocytic pathways.

MVMp cellular uptake occurs in proximity to focal adhesions

We previously reported that MVMp clusters at the leading edge of migrating cells (Garcin and Panté, 2014) and our EM results show that MVMp internalization occurs at the base of filopodia (Fig. 1). Thus, MVMp might be endocytosed in proximity to focal adhesion sites, which play a pivotal role during cell migration. We then investigated the proximity of MVMp clusters to focal adhesion sites by using the focal adhesion markers paxillin and α5-integrin. For these experiments, LA9 and PyMT cells were assayed for MVMp uptake using our short-term MVMp endocytosis assay and prepared for IF microscopy using antibodies for MVMp and paxillin. In another experiment, the short-term MVMp endocytosis assay was performed in cells that were first transfected with α5-integrin-GFP. As shown in Fig. 3A, there was a clear proximity between MVMp clusters and both markers of focal adhesions, indicating that MVMp cellular uptake could indeed take place at contact sites between the cell and the ECM.

To confirm the IF finding that MVMp cellular uptake occurs at cell-ECM contact sites, cells were assayed for MVMp uptake with our short-term MVMp endocytosis assay, prepared for EM, and sectioned vertically (i.e., perpendicular to the monolayer) to allow visualization of focal adhesion sites and ECM. Using this approach, vesicles containing MVMp particles forming at cell-ECM contact sites were visualized (Fig. 3B, right panel). EM analysis of vertically sectioned MVMp-infected cells also revealed vesicles containing MVMp particles at the base of filopodia (Fig. 3B, left panel). Thus, MVMp appears to be endocytosed by migrating cells during turnover of the plasma membrane or focal adhesions, and cellular uptake can occur immediately at the base of filopodia, or from focal adhesion sites.

MVMp uses both clathrin- and lipid-raft mediated endocytosis

Our EM analysis of MVMp-infected cells revealed virions internalized in clathrin-coated vesicles and non-coated pits (Figs. 1 and 2). To verify these observations, we performed our short-term MVMp endocytosis assay with LA9 and PyMT cells, and prepared the cells for IF microscopy using a clathrin-specific antibody. Because some clathrin-independent endocytic pathways are lipid raft-mediated, we also detected lipid rafts by using FITC-conjugated cholera toxin B subunit in these experiments. As illustrated in Fig. 4, MVMp co-localized with both clathrin and cholera toxin B, indicating that MVMp uses CME and lipid-raft mediated endocytosis in both LA9 and PyMT cells.

MVMp uses a variety of endocytic pathways

Our EM results indicate that MVMp internalization occurs via clathrin- and caveolin-carriers, as well as CLICs (Fig. 2). To verify these findings, we investigated MVMp uptake in the presence of various drug inhibitors of these endocytic pathways. CME was inhibited by chlorpromazine, which induces the miss-assembly of clathrin-coated pits at the plasma membrane (Wang et al., 1993). The effect of chlorpromazine on cellular uptake of MVMp was studied using an IF MVMp endocytosis assay in the presence of drugs. For this assay LA9 and PyMT cells were first pretreated with chlorpromazine and bafilomycin A1 (bafA1) for 1 h, then incubated with MVMp at an MOI of 8 for 15 min at 4 °C, and subsequently maintained for 4 h at 37 °C (all in the presence of the drugs). BafA1 inhibits the vacuolar H^+-ATPase in the endosomal membrane that is responsible for acidification (Bayer et al., 1998), and thus bafA1 arrests MVMp in early endosomes, facilitating observation of the internalized virions. As a control, cells were treated with DMSO, instead of chlorpromazine. As documented in Fig. 5, chlorpromazine treatment reduced the MVMp uptake in both LA9 and PyMT cells, but some MVMp immunostaining was still detected in the cytoplasm. This observation supports our findings that cellular uptake of MVMp is via clathrin-dependent route, but also by other endocytic pathways.

Similar MVMp uptake experiments were performed in cells treated with genistein, an inhibitor of caveolae-mediated uptake (Pelkmans et al., 2002). IF microscopy showed that genistein reduced MVMp uptake even though some virions were still found in the cells (Fig. 5). Consistent with our EM analysis (Fig. 2A),

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